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Seasonal Changes in Plasma Growth Hormone and Prolactin Concentrations of the Frog *Rana esculenta*

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Plasma growth hormone (GH), prolactin (PRL), and vitellogenin (VTG) concentrations were determined during the annual reproductive cycle of the frog *Rana esculenta*. Plasma GH and PRL were measured using a RIA that employed purified bullfrog (*Rana catesbeiana*) GH and PRL as standards and radioligand, and their respective antibodies. Using ELISA, plasma VTG titers were related to ovarian weight. GH, PRL, and VTG displayed different trends related to season and sex. In male frogs the GH and PRL trends have been found parallel, showing the highest concentrations (35 and 85 ng/ml, respectively) during the winter months. In the female frogs, the GH trend behaved differently from that in males; in fact, plasma GH changed during the annual reproductive cycle, showing two main peaks occurring during the reproductive period and the autumn ovarian recrudescence that are well correlated with the vitellogenesis as well as with the main changes in ovarian weight. © 1994 Academic Press, Inc.

The study on the differentiation of the anuran anterior pituitary gland, with special regard to the immunochemistry and topographical distribution of growth hormone (GH) and prolactin (PRL), showed the simultaneous appearance of cells immunoreactive to anti-GH and anti-PRL at very early stages of larval development in *Rana dalmatina* (Guastalla and Campantico, 1990). Recently, in *Rana catesbeiana*, Kikuyama (1993) and Kobayashi *et al.* (1989), using both RIA and immunoblot analysis and antisera against bullfrog GH and PRL, found that the embryonic pituitary contains cells in which the granules are immunoreactive with GH and PRL; these authors also ascertained that clearly each antiserum specifically recognizes its antigen. These findings, supported by electromicroscopical observations and immunogold techniques, are consistent with the fact that cells containing granules will stain with anti-GH and at the same time with anti-PRL. Moreover, homologies of nucleotide and amino acid sequences between GH and PRL of the bullfrog origin were ascertained. In fact, Takahashi *et al.* (1990, 1992) showed that homologies of nucleotide and amino acid sequences between GH and PRL were 48 and 26%, respectively.

In amphibians, growth hormone exerts somatotrophic effects on adult animals, while prolactin stimulates the growth of larval organs (Dodd and Dodd, 1976; Kikuyama *et al.*, 1993). It has also been demonstrated that the action of GH is mediated through a serum factor (Kobayashi *et al.*, 1989), as in the case of mammals (van Wyk, 1984). Plasma GH levels are maximal in juvenile frogs and decline as adulthood approaches (Kobayashi and Kikuyama, 1991). On the other hand, PRL is known to be implicated in the developmental, osmoregulatory, and reproductive processes (Bern and Nicoll, 1969), and the occurrence of PRL binding sites in amphibian tissues

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has been reported by several authors; in *Rana esculenta*, the binding of ^{125}I-labeled ovine PRL to membrane preparations of skin, kidney, and liver has been shown (D'Istria *et al.*, 1987). In addition, the water drive effect of PRL has been demonstrated by Vellano *et al.* (1967), Duvall and Norris (1977), and Moriya (1982) in five species from three families of urodele amphibians; conversely, such an effect has never been reported in anuran amphibians. Taken together, these findings show that GH and PRL are involved in the regulation of different types of physiological functions, including reproduction. In this regard, the recent results of Carnevali and Mosconi (1992) and Carnevali *et al.* (1992, 1993) demonstrated the effects of pituitary homogenate and of both GH and PRL in inducing vitellogenin (VTG) synthesis in cultured liver of *R. esculenta*, an anuran common in temperate areas. Frogs of this species, living in the Colfiorito pond (820 m above sea level) and studied for many years in this laboratory, spend a short time in their terrestrial habitat, since they move around for predation only in the summer. The reproductive cycle in this mountain population is characterized by the reproductive period in late spring (May), a postreproductive summer phase, the early autumn recrudescence, and winter stasis. With its well-defined cyclical changes, this frog seemed a good model in which to study GH and PRL involvement in the reproductive function, with special reference to the vitellogenic process which controls the hepatic synthesis of large amounts of the plasma egg protein precursor, vitellogenin, and that is under multihormonal control.

Plasma samples of the wild adult male and female frogs were collected throughout the year, and plasma GH and PRL were measured by RIA using antibodies against *R. catesbeiana* GH and PRL, respectively; an ELISA, suitable for this species, was employed for assessing VTG.

MATERIALS AND METHODS

Animals

A wild population of *R. esculenta* from the Colfiorito (Umbria, Italy) mountain pond at 820 m above sea level was studied from 1991 to 1992, with air and water temperature being regularly recorded at the meteorological station located near the pond.

The total solar radiation, expressed as cal/cm^2, was measured monthly using a pyranometer (Kipp and Zonen, Holland). Ten males and ten females were captured monthly during the years 1991–1992. Each animal was anesthetized by 3-amino benzoic acid ethyl ester (Sigma, St. Louis, MO; 10 g/liter tap water) within 5 min after capture, and blood was immediately collected into a heparinized syringe by cardiac puncture. Blood samples were stored on ice until processed; after centrifugation, plasma was frozen on dry ice and stored at −70° until assay. The animals were weighed, and the gonads were removed and weighed to the nearest milligram.

Vitellogenin (VTG) Assay

Plasma VTG titers were assayed using a modified method of Nunez Rodriguez *et al.* (1989).

Antigen coating. The coating was performed in 96-well microtiter plates (Greiner, medium affinity, SIAL Rome, Italy) in 200 μl of carbonate buffer, pH 9.6, containing 100 ng/ml of VTG. The blank values were obtained by coating eight wells with 100 ng/ml of lyophilized male plasma proteins or bovine serum albumin (BSA, Sigma) at the same concentration. The plates were then covered and incubated for 16 h at 4°. The content of the wells was discarded by inverting the plates, and three successive washes of 30 sec each were applied using 0.01 *M* phosphate buffer (pH 7.4), 0.15 *M* NaCl, and 0.05% Tween 20 (PBS-T). The saturation of nonspecific binding sites was achieved by incubating the plates with 2% normal pig serum in PBS-T (PBS-T-NPS). The plates were maintained for 30 min in an oven at 37° followed by a three-wash cycle with PBS-T.

Specific antibody incubation. In separate tubes the specific antibodies, diluted 1:10,000 in PBS-T-NPS, were preincubated with serial dilutions (factor 2) of samples (diluted at least 1:100) or of reference preparation (VTG from 2000 ng/ml to 12.5 ng/ml) for 16 h at 4°. The content of these tubes was then distributed in duplicates (200 μl/well) and the plates were incubated in plates for 4 h at 37°, followed by a three-wash cycle with PBS-T.

Second antibody incubation. Each well received 200 μl of goat IgG anti-rabbit IgG (Sigma; diluted 1:2000 in PBS-T-NPS). The plate was incubated for 45 min at 37° and rinsed as before.

Peroxidase–anti-peroxidase (PAP) complex incubation. As in the previous step, the PAP complex (Sigma) obtained in rabbits (diluted 1:3000 in PBS-T-NPS) was distributed in the wells, and the plate was incubated for 30 min at 37° and then washed.

Initiation of the reaction. Each well received 200 µl of the following solution prepared immediately before use: 20 ml of 0.1 *M* citrate–phosphate buffer (pH 5), containing 10 mg of *o*-phenylenediamine (Sigma), and 10 µl of 30% hydrogen peroxide.

Color development reached its maximum after 15 min in the dark at room temperature (20°), and the reaction was stopped by adding 50 µl/well of 4 *M* sulfuric acid. The absorbance of each well was measured at 492 nm using a Titertek EIA reader.

Assay validation. The working conditions were determined to obtain a maximum absorbance value (B_o) near 1.5, since the plate EIA reader gave linear responses in the range of 0–2.0 o.d. units.

The sensitivity (VTG amount which gave 90% of the binding) was about 3 ng/well with an intraassay variation of 5% ($n = 20$) and an interassay variation of 7.8% ($n = 12$) around 50% of the binding.

Radioimmunoassay (RIA)

Plasma PRL was measured by an RIA employing purified bullfrog (*R. catesbeiana*) PRL (fPRL) (Yamamoto and Kikuyama, 1981) as standard and radioligand, and its antibody. The radioiodination of fPRL was performed by the lactoperoxidase method (Yamamoto and Kikuyama, 1982). Lyophilized plasma samples were reconstituted with distilled water at a concentration twice as high as the original plasma samples, and then diluted with diluent (1% BSA–PBS, pH 7.5) containing 1% normal rabbit serum to an appropriate concentration. For measuring PRL in the plasma, the antiserum was used at a final dilution of 50,000, at which the antiserum bound about 30% of the label in the absence of any unlabeled hormone. Other details of RIA procedures were the same as those used in the fPRL RIA (Yamamoto and Kikuyama, 1982).

GH in the plasma of *R. esculenta* was also measured by an RIA using purified bullfrog (*R. catesbeiana*) GH (fGH) (Kobayashi *et al.*, 1989) as standard and radioligand, and an antiserum to fGH. In this RIA, the buffer used was 0.01 *M* phosphate buffer (pH 7.3) containing 0.14 *M* NaCl, 1% BSA, 0.1% Triton X-100, and 0.1% NaN_3. The antiserum to fGH was used at a final concentration of 1:50,000. All assay tubes were incubated for 24 h at 4°. After incubation, 0.2 ml of goat antiserum to rabbit γ-globulin and 10% polyethylene glycol were added and incubated for 24 h at 4°. After the second antibody reaction was completed, the tubes were centrifuged and the precipitate was counted in a gamma counter. Other details were as described previously (Kobayashi and Kikuyama, 1991).

The inhibition curve of *R. esculenta* plasma was parallel both to that of fGH and to the fPRL standard. Statistics for linearity and parallelism in the parallel line assay were computed according to the method of Bliss (1952).

Statistical Analysis

Results were analyzed with a statistical software package, Stat View 512 (Brain Power Inc., USA), operating on a Macintosh Plus computer (Apple, USA). A probability level of 0.05 was taken to indicate a statistical difference between means. Results are expressed as means ± SE of data.

RESULTS

Figure 1 shows the competition curve in the VTG assay; parallel displacement was obtained with the standard preparations and serial dilution of female frog plasma. As shown in Fig. 2, the slope of the inhibition curve produced by *R. esculenta* plasma was parallel to that of the bullfrog (*R. catesbeiana*) GH (Fig. 2a) and PRL (Fig. 2b) standards.

Field Observation

Observations of this frog population over the years 1990 and 1991 revealed no considerable year-to-year variations in the timing of the annual cycle. The changes of air and water temperatures are consistent with warm temperatures in May when spawning occurs, and one- or two-egg depositions were observed; in addition, the most important increase in the monthly variation of solar radiation occurred in May, as reported in Fig. 3. Breeding is interrupted in the summer months.

Males

In the male frog plasma GH and PRL (Fig. 3) show a similar trend, since the lowest GH levels were found between March and September (about 8–15 ng/ml), and baseline levels of PRL (about 2 ng/ml) were found during the same period. After that, GH titers rose significantly ($P < 0.01$) in

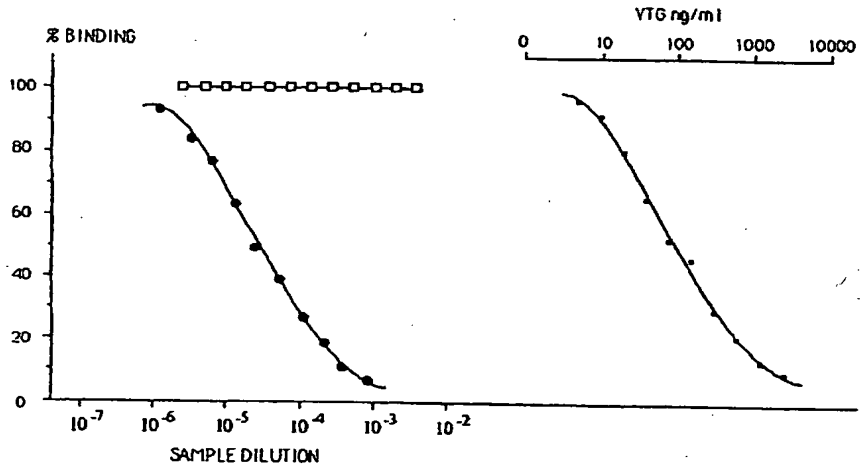

FIG. 1. Binding curves (log (dose) = $f(B - N/B_0 - N)\%$) obtained with the following antigens: *Rana* VTG reference preparation (■), serial dilutions of female (●) plasma. Serial dilutions of male plasma (□) did not give any significant displacement of binding in this system. Parallelism of the regression curve was assessed by the F test on mean squares. The F test did not reveal significant differences between the regression curve of competing antigens.

October, as did those of PRL in December ($P < 0.01$). The highest levels of GH and PRL were found during the winter months, since the decreases of both GH and PRL levels found in January were not statistically significant. The winter peak values of GH and PRL were followed by sharp decreases just before the beginning of spring (March).

Females

The female GH trend during the annual reproductive cycle differs from that of males; in fact, plasma GH (Fig. 4, upper panel) was found to peak in May (about 50 ng/ml) and decrease ($P < 0.01$) suddenly in June, remaining at low levels during the summer months until September. In October, plasma GH titers increased ($P < 0.05$), remaining around this value (25 ng/ml) during the winter months; the decreased GH observed in February and March was not statistically significant. The changes of plasma VTG mirrored those of plasma GH described above (Fig. 4, upper panel). In fact, the plasma VTG titers, during the annual reproductive cycle, assayed in the same plasma samples in which GH and PRL were tested, varied according to the ovarian weight (GSI) changes and showed two significant peak values: one in April, at the beginning of the reproductive phase, and the other in the autumn recrudescence (September). Both VTG increases were statistically significant ($P < 0.01$) as were the increasing values of GSI ($P < 0.05$) found at the same time. With regard to female PRL, increasing amounts were found, as in males, during the winter months. The baseline levels of plasma PRL (Fig. 4, upper panel) were found from March until November; a very significant ($P < 0.01$) peak value of 25 ng/ml in December was followed by weak decreasing values in January and February, the baseline levels being reached in March.

DISCUSSION

In amphibians, it is well known that GH and PRL participate in a wide variety of

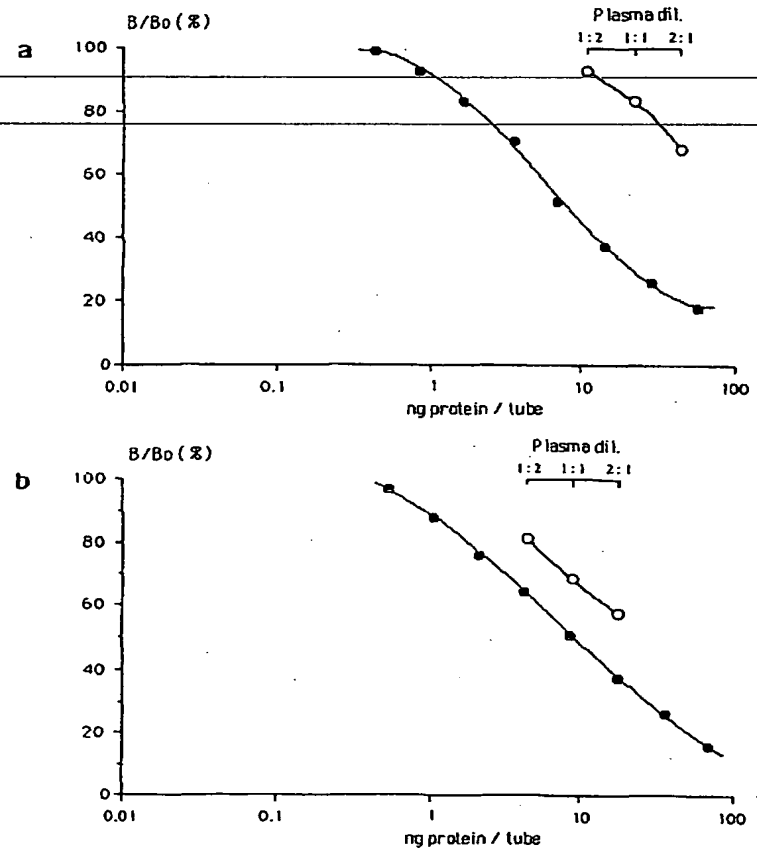

FIG. 2. (a) Displacement of ^{125}I-fGH (●) with serially diluted plasma (○) of *Rana esculenta*. (b) Displacement of ^{125}I-fPRL (●) with serially diluted plasma (○) of *Rana esculenta*.

physiological functions. The present findings concern the involvement of these two hormones in the reproductive biology of the anuran species *R. esculenta*, with special reference to the vitellogenic process. The annual changes of plasma GH and PRL levels in this mountain population of the frog revealed high seasonality. In males a similar trend of these two hormones was apparent. Conversely, in females the annual plasma changes of GH did not parallel those of PRL. Prolactin, as in males, proved to be maximal during winter months, while a GH peak was found not only in winter, but also in May. With regard to the function of PRL in amphibians, useful suggestions coming from the investigations conducted in depth by Ishii *et al.* (1989) and by Yamamoto *et al.* (1989) are consistent with the possible role of PRL in toad osmoregulation in water, and/or oviducal jelly secretion in the female. The results obtained by these authors also indicated that PRL is not a water drive factor in *Bufo japonicus*, even if this function has been well established in most urodele species. *B. japonicus* was a good model in which to assess the role of PRL as a water drive factor, since it is a common terrestrial toad in Japan that spends only a few days in

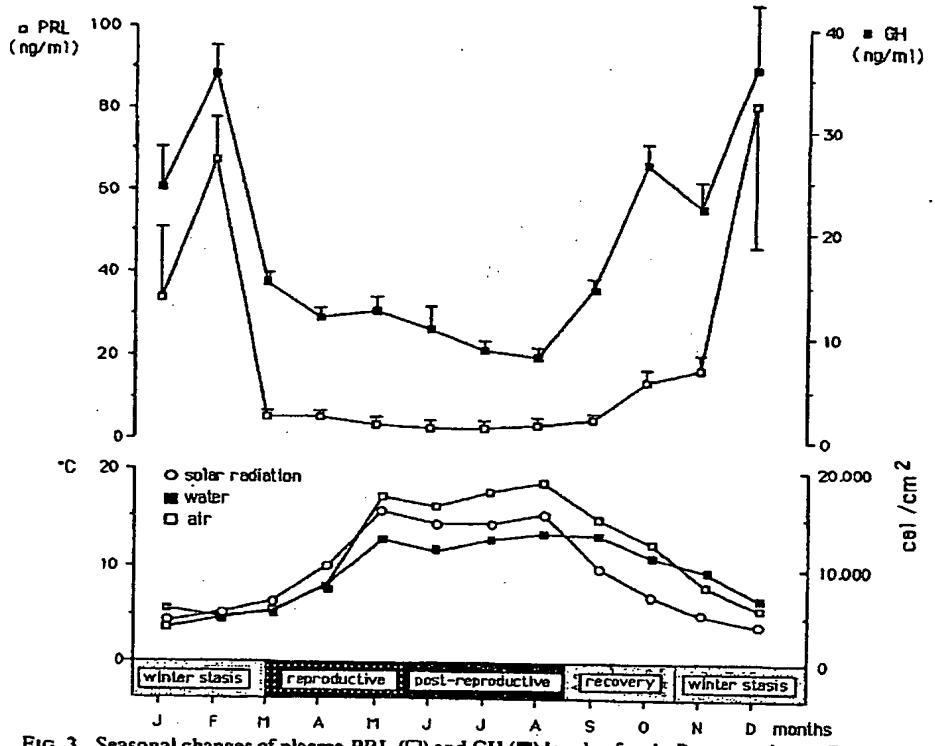

FIG. 3. Seasonal changes of plasma PRL (□) and GH (■) levels of male *Rana esculenta*. Each value is the mean ± SE of 10 determinations (upper panel). Seasonal changes of solar radiation and air and water temperatures in Colfiorito pond (lower panel).

its aquatic habitat during the short breeding period in early spring. In this respect, the frog *R. esculenta*, studied here, seems a useful model too, since it is mainly aquatic and only for a short time, during summer months, can animals be captured on land. The higher levels of plasma PRL in both male and female *R. esculenta* found during the winter months open up additional undefined actions of PRL in the anuran reproductive physiology. In fact, the highest plasma PRL levels are not correlated with the breeding period; for that reason, the suggestion of Ishii *et al.* (1989) that PRL is associated with the highest plasma androgen levels and reproductive behavior in males does not concur with the present data nor with the possibility that PRL induces jelly secretion in females. Our data show the highest plasma PRL levels during winter months, a significant increase starting, in both males and females, in October when a sharp drop in water temperature occurred in the mountain pond. It is thus suggested that PRL may play a regulatory role in adaptation to environmental conditions, at least in *R. esculenta*. Regarding GH actions in the amphibian, the somatotrophic effects on adult individuals (such as hind limbs) have been well documented, as well as its involvement in facilitating the rapid growth of juvenile frogs, since GH mRNA levels reached a maximum in the juvenile individuals (Takahashi *et al.*, 1992). It has also been shown that GH secretion in amphibians is modulated by thyrotrophin-releasing hormone (TRH) acting directly upon the GH cell (Gracia-Navarro *et al.*, 1991). The

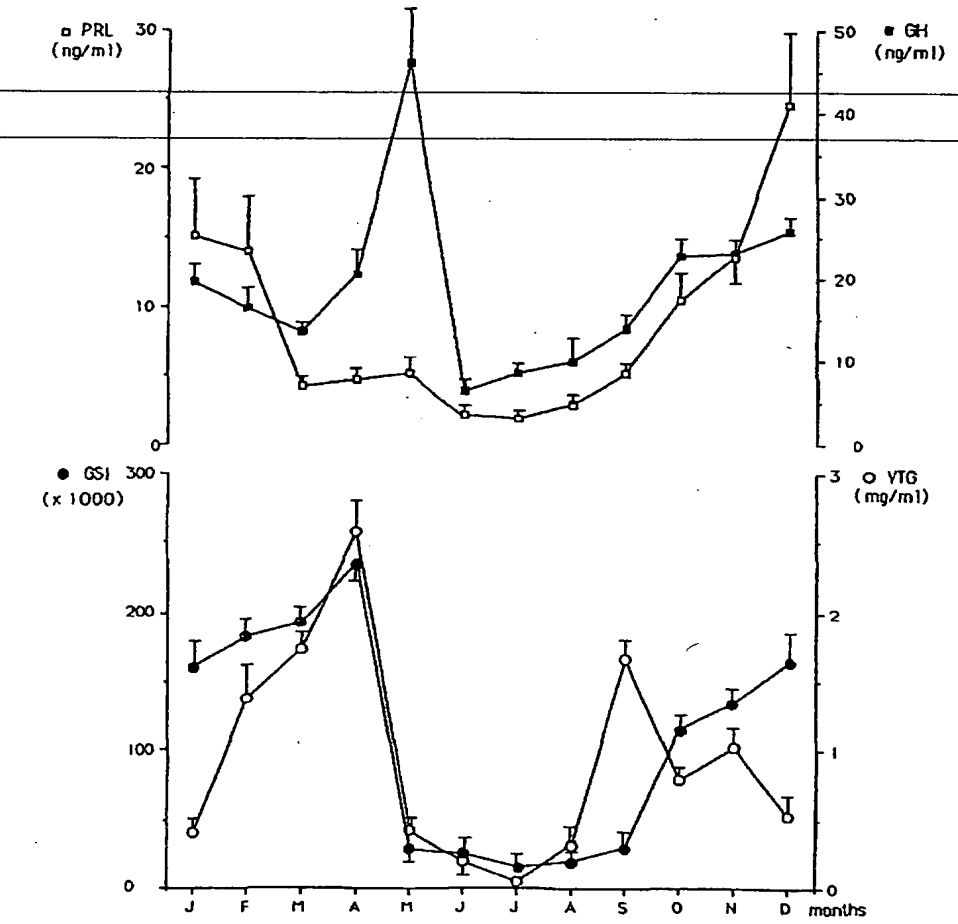

FIG. 4. Seasonal changes of plasma PRL (□) and GH (■) (upper panel); plasma VTG (○) and GSI (●) (lower panel) of female *Rana esculenta*. Each value is the mean ± SE of 10 determinations.

possible involvement of GH in amphibian reproduction, especially in vitellogenesis, comes from the data obtained by Carnevali *et al.* (1992) on the action exerted by bullfrog GH on the induction of VTG synthesis in cultured livers of *R. esculenta*. Previously, *in vivo* experiments in both anuran and urodele amphibians, as well as in other oviparous vertebrate species, suggested that hormones other than estradiol-17β regulate vitellogenic processes. In this way, pituitary hormones have been considered reasonable candidates.

The GH trend in female frogs, reported here, is different from that in males, whose GH annual profile paralleled that of the PRL. Conversely, the GH female plasma changes during the reproductive annual cycle are consistent with the two main peaks: the first one occurring at the breeding period and the second during autumn ovarian recrudescence; during that period it has been previously shown by Giorgi *et al.* (1982) that vitellogenic processes work in this frog to induce the main representative changes of ovarian weight. Even if the two plasma VTG peaks are not contemporaneous with the GH ones, these *in vivo* exper-

iments supplement the previous findings about the involvement of GH in vitellogenic processes; the reason for this is that the plasma VTG concentration should be considered as a balance between that synthesized by liver and that taken up by the ovary.

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Vitellogenin as a Biomarker of Exposure for Estrogen or Estrogen Mimics

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Abstract. Vitellogenin, the egg yolk precursor protein, has become a popular biomarker for measuring exposure of oviparous animals to estrogen or estrogen mimics. Vitellogenin is normally produced by females in response to normal cycles of estradiol during oogenesis. The gene for vitellogenin is also present in the livers of males but it is normally silent. Upon exposure to estrogen or to an estrogen mimic, the gene is turned on and vitellogenin is synthesized. After synthesis, it is exported into the blood where, in males, it remains until it is degraded or cleared out by the kidneys. In females, vitellogenin is taken up by the developing oocyte through receptor mediated endocytosis.

There are several assays in the literature for measuring vitellogenin levels in plasma. The easiest method is through antibody based assays including ELISA (enzyme-linked immunosorbent assay) or by western blot. Competition or sandwich ELISAs are the most sensitive assays and they can detect vitellogenin in plasma in the nanogram to milligram per ml range.

This chapter discusses methods for purifying vitellogenin from plasma, generating antibodies, and performing assays to measure vitellogenin.

Keywords: estrogen, endocrine disruption, biomarker, vitellogenin

CONTENTS

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1. Introduction

Vitellogenin (Vtg) is an ideal biomarker for measuring exposure of oviparous animals to estrogen or to estrogen-mimicking compounds. Vtg is a large (300–600 kDa native 160–200 kDa subunit), serum phospholipo-glycoprotein that serves as the major precursor to the egg-yolk proteins of oviparous vertebrates (Wallace, 1985). The gene for Vtg is found in the liver of both females and males, where it is activated by exposure to estrogen. Vtg is normally only synthesized in mature females, where the level of estrogen is above the threshold required to induce it. Vtg is secreted into the serum where it circulates till it reaches the developing oocyte which takes it up by receptor-mediated endocytosis. Vtg levels in the range of 10–20 mg ml have been noted at peak activity in plasma of females (Denslow et al., 1997b; Folmar et al., 1996; Parks et al., 1999).

Males normally do not synthesize Vtg. But, upon exposure to estrogen or an estrogen mimic, they will be induced to synthesize Vtg, since they have the gene for it in their livers. Vtg, with no where to go, accumulates in the plasma until it is degraded by plasma proteases. Very high levels of Vtg in males have been linked with kidney failure (Folmar et al., unpublished observation). Thus, finding Vtg in males is an indication of exposure to an estrogenic substance (Sumpter and Jobling, 1995). Some contaminants, acting as anti-estrogens, may show their effects in females by interfering with the natural production of Vtg. In this case, lower than normal levels of Vtg would be found in the plasma.

The potential of Vtg as a biomarker has already been explored using several fish species for which both in vivo and in vitro assays have been developed (Denslow et al., 1997a, 1997b; Folmar et al., 1996; Heppell et al., 1995; Jobling and Sumpter, 1993; Pelissero et al., 1993; Sumpter and Jobling, 1995). Because Vtg functions as a nutritional source for the developing embryo, rather than as an important functional protein, its primary sequence has not been conserved among species (Wahli et al., 1981; Lee et al., 1992; Carnevali and Belvedere, 1991). Thus, except for short patches of homology along the entire molecule, Vtgs from different species share little structural similarity. For this reason, an antibody made against Vtg from one species is limited in its application as a probe for another. But, because there are some segments of Vtg that appear to be highly conserved among species, it is possible to develop antibodies with wide cross-reactivity among species and these may serve a more universal role (Folmar et al., 1995; Heppell et al., 1995, Denslow et al., 1997b).

It has been estimated that Vtg mRNA synthesis can be induced as much as 40,000 fold over background levels (Pakdel et al., 1991). This large induction of mRNA gives rise to as much as 100 mg ml protein in some species, for example sheepshead minnow, when they are injected with pharmacological levels of estradiol. The natural induction varies from fish to fish and is probably at least one order of magnitude less. Vtg induction can be measured both at the mRNA level and at the protein level. These measurements are complementary to each other. Measurements made at the mRNA level indicate a recent exposure to estrogen, because the mRNA is quickly degraded with a half life of about 2 days (unpublished observations). These measurements are sensitive, responding quickly (within 6 hours) to low levels of estradiol, 20–100 ng L. Measurements, on the other hand, made at the protein level, may indicate exposure has occurred over a longer time interval. Vtg protein levels remain high in the plasma for some time after the exposure, the protein degrades much more slowly than the mRNA.

1. Introduction

Vitellogenin (Vtg) is an ideal biomarker for measuring exposure of oviparous animals to estrogen or to estrogen-mimicking compounds. Vtg is a large (300–600 kDa native 160–200 kDa subunit), serum phospholipo-glycoprotein that serves as the major precursor to the egg-yolk proteins of oviparous vertebrates (Wallace, 1985). The gene for Vtg is found in the liver of both females and males, where it is activated by exposure to estrogen. Vtg is normally only synthesized in mature females, where the level of estrogen is above the threshold required to induce it. Vtg is secreted into the serum where it circulates till it reaches the developing oocyte which takes it up by receptor-mediated endocytosis. Vtg levels in the range of 10–20 mg ml have been noted at peak activity in plasma of females (Denslow et al., 1997b; Folmar et al., 1996; Parks et al., 1999).

Males normally do not synthesize Vtg. But, upon exposure to estrogen or an estrogen mimic, they will be induced to synthesize Vtg, since they have the gene for it in their livers. Vtg, with no where to go, accumulates in the plasma until it is degraded by plasma proteases. Very high levels of Vtg in males have been linked with kidney failure (Folmar et al., unpublished observation). Thus, finding Vtg in males is an indication of exposure to an estrogenic substance (Sumpter and Jobling, 1995). Some contaminants, acting as anti-estrogens, may show their effects in females by interfering with the natural production of Vtg. In this case, lower than normal levels of Vtg would be found in the plasma.

The potential of Vtg as a biomarker has already been explored using several fish species for which both in vivo and in vitro assays have been developed (Denslow et al., 1997a, 1997b; Folmar et al., 1996; Heppell et al., 1995; Jobling and Sumpter, 1993; Pelissero et al., 1993; Sumpter and Jobling, 1995). Because Vtg functions as a nutritional source for the developing embryo, rather than as an important functional protein, its primary sequence has not been conserved among species (Wahli et al., 1981; Lee et al., 1992; Carnevali and Belvedere, 1991). Thus, except for short patches of homology along the entire molecule, Vtgs from different species share little structural similarity. For this reason, an antibody made against Vtg from one species is limited in its application as a probe for another. But, because there are some segments of Vtg that appear to be highly conserved among species, it is possible to develop antibodies with wide cross-reactivity among species and these may serve a more universal role (Folmar et al., 1995; Heppell et al., 1995, Denslow et al., 1997b).

It has been estimated that Vtg mRNA synthesis can be induced as much as 40,000 fold over background levels (Pakdel et al., 1991). This large induction of mRNA gives rise to as much as 100 mg ml protein in some species, for example sheepshead minnow, when they are injected with pharmacological levels of estradiol. The natural induction varies from fish to fish and is probably at least one order of magnitude less. Vtg induction can be measured both at the mRNA level and at the protein level. These measurements are complementary to each other. Measurements made at the mRNA level indicate a recent exposure to estrogen, because the mRNA is quickly degraded with a half life of about 2 days (unpublished observations). These measurements are sensitive, responding quickly (within 6 hours) to low levels of estradiol, 20–100 ng L. Measurements, on the other hand, made at the protein level, may indicate exposure has occurred over a longer time interval. Vtg protein levels remain high in the plasma for some time after the exposure, the protein degrades much more slowly than the mRNA.

1.1. Vitellogenin protein assays

Vitellogenin has been detected in plasma by a number of different methods, including radio-immunoassays, enzyme-linked immunosorbent assays (ELISA) and western blotting methods (Campbell and Idler, 1980; Denslow et al., 1997a, 1997b; Folmar et al., 1995; Heppell et al., 1995; Mananos et al., 1994; Nunez-Rodriguez et al., 1989; Specker and Anderson, 1994). Each of these methods have advantages and disadvantages. The easiest and most automated assays are designed around ELISA methods. However, prior to using these methods, one must validate that the antibody is specific for Vtg by some independent method, such as by western blot analysis or by immuno-precipitation and protein sequence analysis. Western blot analysis is very specific because one can observe the binding of the antibody to the correct size protein after separation of proteins by size, but it is difficult to quantify and cumbersome to perform for a large group of samples. Because of these considerations, most laboratories use ELISA.

There are several different types of ELISAs that can be performed including a **direct assay** (plasma containing Vtg is adsorbed directly to the microtiter plate), a **competition assay** (a known amount of control Vtg adsorbed to the plate competes with Vtg in plasma samples for antibodies, and a **sandwich assay** (antibodies are adsorbed to the plate and they bind Vtg in the plasma). Of these methods, the direct assay is the easiest to perform, but the least sensitive. Competition assays and sandwich assays are both very sensitive (down to 1 ng ml Vtg) and about equally cumbersome to perform.

One can use either monoclonal or polyclonal antibodies directed against Vtg. There are advantages and disadvantages for each type of antibody preparation. Polyclonal antibodies are easier to prepare and provide higher signal to noise ratios, thus are more sensitive than monoclonals, but they may be less specific and harder to validate. Monoclonals, on the other hand, are very specific, each to a single epitope, but they are more expensive to obtain and may be less sensitive. In this chapter we will describe methods to obtain both polyclonal and monoclonal antibodies, assays for their validation and provide protocols for direct and sandwich ELISAs and for Western blotting.

2. Preparation of reagents

As with most procedures, the best results will be obtained with purified and well characterized reagents. For ELISAs one must have specific antibodies (either polyclonal or monoclonal) and well characterized, pure species-specific Vtg for a standard curve, and in the case of a competition assay, enough Vtg to coat the bottoms of microtiter plates.

2.1. Estradiol-stimulation of animals to produce vitellogenin

Vtg is inducible by estradiol. Normally, one can inject fish with 1–5 mg Kg 17—8 estradiol in corn oil or DMSO. Maximum stimulation is achieved with two injections, one week apart. It is important to collect a sample of male control plasma either from the same animal, prior to the first injection, or from other animals.

1. Materials required:
   17—8 estradiol (1,3,5,(10)-estratrien-3,17 diol) (Sigma, catalog #E-8875)
   Corn oil
   Ethanol (70%)
   Syringes (1–5 ml)
   Vacutainer needles and holder (22 Ga-1, Becton Dickinson 800-631-0174)
   Vacutainers (heparanized, 3 ml, Becton Dickinson 800-631-0174)
   MS-222 (tricaine methane sulfonate)
   Labels (tough tags USA Scientific Plastics)
   Microcentrifuge tubes (1.5 ml)
   Protease inhibitor—aprotinin (Sigma A-6279) make a 1000X stock solution which is 10 KIU μl in 150 mM NaCl.
   Use male animals if at all possible
2. Prepare 17—8 estradiol in corn oil. The injection dosage should be 1–5 mg Kg. Injection volumes should range from 100–500 μl per animal for large fish (> 100 g). For small fish (100 g), inject 10–50 μl volume per animal. Weigh each animal to estimate size. Make a stock solution of estradiol in a small

volume of ethanol and then mix in with corn oil. Vortex well.
3. Lightly anesthetize animal with 50–100 ppm MS-222.
4. Inject animal interperitoneal (IP) with a small volume (50–500 μl) of estrogen oil mixture to give a final dose of 1–5mg kg. Choose a posterior injection site that is away from organs.
5. Collect blood 2 weeks after estrogen injection.
6. Lightly anesthetize animal with MS-222 as discussed above.
7. Insert needle into a vein (caudal vein for fish), insert heparinized vacutainer, collect blood, add aprotinin to a final concentration of 10 KIU ml, gently mix blood with heparin by inverting 2 times and then keep cool at 4°C. Euthanize fish (or animal) after collection.
8. Centrifuge blood for 15–20 min at 4,000 × G at 4°C to obtain plasma.
9. Transfer plasma into a separate labeled microcentrifuge tube. Label.
10. Store at 80°C.

2.2. *Purification of vitellogenin*

There are several ways to purify Vtg's that rely on the physical-chemical properties of this unusual protein (Hara et al., 1993, for review of several methods see Specker and Sullivan, 1994). A direct approach is to bind Vtg to an anion exchanger, such as DEAE agarose (Parks et al., 1999; Heppell et al., 1999). Vtg can be further purified by size exclusion chromatography since very few plasma proteins are in the size range, 300–600 kDa, of Vtg (Specker and Sullivan, 1994; Kroll and Doroshov, 1991).

The method we describe in this protocol is based on binding Vtg to a strong anion exchanger affixed to a perfusion chromatography bead (Poros 20 HQ) at conditions under which most plasma proteins do not bind, pH 9 and 150 mM salt. This procedure is rapid and yields Vtg that remains largely intact and is at least 98% pure, as determined by SDS PAGE.

Vtg is normally a large protein (540 kDa on native gels and 160–200 kDa on SDS-PAGE). It is very susceptible to proteolytic degradation, thus it is wise to include antiproteolytic agents such as aprotinin and PMSF. Even with these agents present, Vtg will degrade. Thus, for best results, purify Vtg soon after obtaining the sample. Vtg is a large lipo-glyco protein—complexed with calcium. It can easily precipitate out of solution if the salt concentration falls below 150 mM. In fact, some researchers use water to salt it out of solution as a preparation method (Specker and Sullivan, 1994). This may not work for Vtg from all sources, however.

1. Reagents and Materials
   Poros 20 HQ, (Strong anion exchanger, PerSeptive Biosystems)
   High flow rate Chromatography system
   Buffer A: 20 mM Tris-bis propane, 150 mM NaCl, pH 9.0
   Buffer B: 20 mM Tris-bis propane, 0.8 M NaCl, pH 9.0
   Column cleaning buffer, 20 mM Tris-bis propane, 2M NaCl, pH 9.0
   Acrodisc 0.45 μm
   Estrogen induced or vitellogenic plasma (0.4 ml)
   Control male plasma (0.2 ml)
2. Pack column with poros 20 HQ resin, following manufacturer's suggestion.

Note: Poros HQ is a strong anion exchanger, similar in quality to MonoQ resin available from Pharmacia.

3. Equilibrate column with 10 column volumes of Buffer A.
4. Dilute plasma sample 1:5 with buffer A to reduce salt and to adjust pH. Ensure that precipitation of Vtg has not occurred. Filter solution through an Acrodisc 0.45 μm.
5. With fast flowing pumps, 10 ml min, inject 2 ml of diluted sample onto column.
6. Wash column with an additional 5 column volumes of buffer A, until baseline returns to background values.
7. Start gradient using buffer B. This will require at least 15 column volumes.
8. After samples are eluted from the column, pool fractions and adjust pH to 7.0 with 500 mM bis-tris propane, pH 6.5. Usually 30 μl per ml fraction of adjust buffer is sufficient. Add 10 μl of aprotinin stock solution to inhibit proteases. Mix with glycerol at a ratio of 1:2.

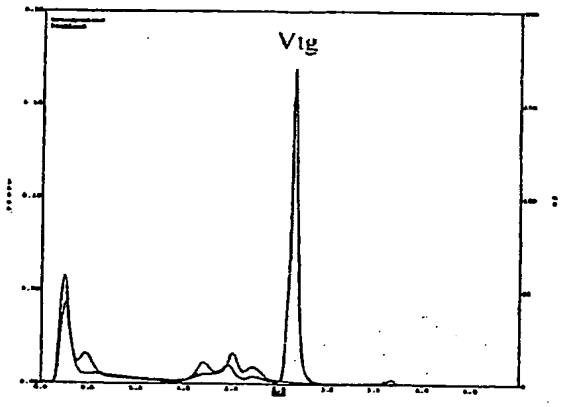

Figure 1. Separation of Vtg from plasma proteins by chromatography on Poros 20HQ. Solid line is plasma from an E_2-induced fish and marked line is plasma from a control male.

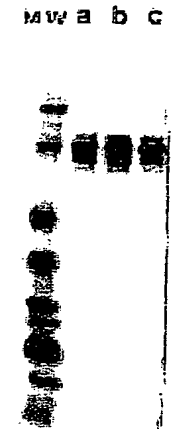

Figure 2. Example of purified Vtg samples from carp obtained by the above procedure analyzed on a Novex 4–20% Tris-glycine SDS gel. Lane MW, molecular weight markers from Novex (Myosin, 250 kDa; Phosphorylase B, 148 kDa; Glutamic dehydrogenase, 60 kDa; carbonic anhydrase, 42 kDa; Myoglobin-Blue, 30 kDa; and Myoglobin-red, 22 kDa), lanes a–c, three different preparations of carp Vtg.

9. Repeat experiment with control male serum to confirm the identity of Vtg peak as female-specific (Figure 1).
10. Analyze fractions of the eluted peak by SDS-PAGE, using acrylamide gels (either Laemmli gels (Laemmli 1970) or Tris-tricine gels (Schagger and von Jagow, 1987) to determine the molecular weight of the eluted material and to confirm purity of the sample. (See Figure 2.)
11. Determine concentration of Vtg by a protein assay such as the Bradford assay.
12. Aliquot, label and store at 20°C.

Note: if storing Vtg aliquots for ELISA standards, each vial should contain about 10 μg protein. Do not freeze-thaw the purified Vtg samples repeatedly. This will cause Vtg to degrade. Vtg in 50% glycerol will not freeze at 20°C, but will at 80°C.

2.3. Preparation of polyclonal antibodies against vitellogenin

Vitellogenin is a highly immunogenic protein, such that when it is injected into rabbits or mice, it elicits a very strong immune response. Prebleeds should be obtained from mice or rabbits to test for crossreacting antibodies. There are several commercial companies that can be contacted for this purpose. Rabbits should be immunized with highly purified Vtg in order to have the best antibody preparation. If Vtg is contaminated with plasma proteins, then the resulting polyclonal antibodies will need to be purified either by affinity chromatography on a column of Vtg or by removing antibodies to other plasma proteins by immuno-precipitation with male control plasma.

1. Reagents and Materials
   2–3 New Zealand white rabbits
   Purified Vtg 400 μg ml (> 98% purity)
   Freund's complete adjuvant (Sigma, St. Louis, MO)
   Freund's incomplete adjuvant (Sigma, St. Louis, MO)
   Phosphate Buffered Saline (PBS): 0.01 M sodium, phosphate, 0.15 M NaCl, pH 7.3
   Centrifuge
2. Immunization protocol
   Mix 0.5 ml Vtg (100 μg) with two volumes Freund's complete adjuvant for the initial inoculation.
3. Inject each rabbit subcutaneously with antigen mixture at multiple sites.

4. Prepare 50 μg of purified Vtg in two volumes incomplete Freund's adjuvant. Repeat injections at 2 week intervals for 2 months, or until the rabbits respond with an appropriate titer.
5. Take test bleeds from the rabbit after each month to test for reactivity to Vtg by ELISA or Western blot.
6. When the antibody titer is judged to be sufficiently high, euthanize and exsanguinate rabbits.
7. Allow blood to clot overnight at 4°C.
8. Centrifuge at 12,700X g to separate the serum. Store at 80°C.
9. Test quality of antibody by ELISA and Western blot to determine if there are any cross-reacting antibodies to other serum proteins.

Note: In the event that there are antibodies that cross-react with other serum proteins, these antibodies can be removed from the antisera by immunoprecipitation with control male plasma obtained from the same species.

2.4. *Preparation of monoclonal antibodies*

Monoclonal antibody refers to an antibody preparation that is secreted by cloned hybridoma cells and is to a single epitope. This is different from the antibody preparation obtained from serum from a rabbit or mouse that has been injected with an antigen. In this case, the preparation is termed polyclonal because it really consists of a mixture of a large number of different antibodies, only a few of which are specific for the antigen used to immunize the animal.

In the animal, antibodies are synthesized individually by different cells, either terminally differentiated B lymphocytes in the plasma or by spleen cells. To make monoclonal antibodies, an antibody secreting cell isolated from the spleen of an immunized animal is fused with a myeloma cell. The hybrid cells, called hybridomas, are immortal and can be grown in vitro. They secrete antibodies with defined specificities. The antibodies are called monoclonals.

Monoclonal antibodies are best made in a Hybridoma Core Laboratory that is routinely set up for this type of work. There are several laboratories that do this on a commercial basis. The procedure takes about 3–6 months from start to finish.

1. Reagents and Materials
   Balb C mice
   Purified Vtg in phosphate buffered saline (Purification need only be > 80%) MPL and TDM emulsion from Ribi Immunochemicals
2. At least 3 Balb C mice should be immunized subcutaneously at three sites with 5 to 20 μg of purified VTG in sterile phosphate buffered saline mixed 1:2 with MPL and TDM emulsion (Ribi).
3. Boost mice twice at 2 to 3 week intervals with the same antigen emulsion.
4. Collect blood from the tail and test by ELISA against Vtg to determine whether the titer of polyclonal antibodies is high. Compare to normal mouse serum.
5. Four days before fusion, boost mice again at one intraperinoneal site with 100 μg antigen with no adjuvant. The best responding mouse is sacrificed and the spleen is prepared for fusion. Monoclonal antibodies are then generated using standard methods (Harlow and Lane, 1988).
6. Screening hybridoma colonies
   Prepare a solution of 2–5 μg Vtg ml. Use 50 μl per well to coat the sides and bottoms of microtiter plates. Test each tissue culture supernatant for antibodies that bind to the antigen by ELISA and by Western blotting (see protocols 6 and 8, below).

Note: After fusing spleenocytes with myeloma cells, the hybrids are seeded out into individual wells of microtiter plates. Normally 500 different wells are seeded. As the cells grow in HAT selective media, hybrids secrete antibodies into the tissue culture supernatant. The cells will be ready for screening approximately 10 to 14 days after the fusion. Samples of tissue culture fluid are removed and tested against the antigen of interest by direct ELISA (see protocol # 8) to determine which have the desired specificity. Only wells containing antibodies that test positive for Vtg should be kept. If the Vtg preparation used to immunize the mouse was not pure, then a second screen using the Western blotting technique is advisable to be sure that the antibodies are recognizing Vtg rather than some other

plasma protein. Tissue culture supernatant can be stored at 4°C in the presence of 0.01% NaN_3 for up to 3 months till use.

7. Positive colonies are subjected to single cell cloning procedures to produce monoclonal cell lines secreting mAbs with desired characteristics. Tissue culture supernatants are screened again by direct ELISA as described in step 6 above.
8. Selected monoclonal cultures are iso-typed using commercial kits (Roche). Select those that produce IgG antibodies.
9. Tissue culture supernatants can be used directly or the monoclonal antibodies (mAbs) can be purified from the supernatants by chromatography on protein G-sepharose.

Note: Supernatants used for purification must be prepared in either serum free media or media supplemented with serum that is IgG free.

2.5. *Purification of monoclonal antibodies by affinity chromatography on Protein G*

1. Reagents and Materials
   Tissue culture supernatant or ascites containing monoclonal antibodies
   Chromatography work station
   POROS 20G (Protein G, PerSeptive Biosystems) or equivalent
   0.02 M Sodium phosphate buffer, pH 7.0
   0.05 M Glycine, pH 2.5
   Acrodisc 0.45 um filter
   0.5 M Tris buffer, pH 6.5
   1% Sodium azide
2. Prepare sample and filter through an Acrodisc 0.45 um filter.
3. For bulk purification use a large column containing 1.5 g of resin.
4. Equilibrate column with 10 column volumes of phosphate buffer.
5. Load 500 ml of culture supernatant onto the column.
6. Wash the column with 10 column volumes of phosphate buffer, until absorbance is back down to the baseline.
7. Save the material that flows through to run again.
8. Switch to 0.1 M Glycine, pH 2.5 and collect fractions of about 1 ml each.
9. Pool peak fractions and adjust to pH 7.0 using Tris buffer (0.5 M, pH 6.5), add azide to 0.05%.

Note: If you are planning to biotinylate the monoclonal antibody, do not add azide to the preparation as this interferes with biotinylation.

10. Determine total protein using a Bradford assay.
11. Aliquot into 1.0 ml fractions, label and store at 80°C.
12. Check purified mAb by gel electrophoresis (12% acrylamide gel) and activity by Western blot or ELISA.

2.6. *Validation of antibody preparation*

In order to be certain that the prepared antibody is in fact against vitellogenin, it is critical to determine this empirically. Several avenues for validation exist, some more definitive than others. The minimal validation experiment should include a Western blot that shows the antibody reacts with a high MW protein (160–200 kDa) present in plasma of females or E_2-induced males and does not cross react with plasma proteins from control males—protocol 6. A more definitive validation would include an immunoprecipitation experiment that clearly shows that the antibody binds specifically to vitellogenin. This can be proven by amino acid sequence analysis of the protein precipitated by the antibody. Amino acid sequence analysis can be performed by Protein Chemistry Core Facilities, available at most major Universities.

1. Reagents and Materials
   Plasma from E_2-induced male or vitellogenic female
   Control plasma from normal male
   2X Laemmli sample buffer (LSB): 125 mM Tris, pH 6.8; 4% SDS; 0.2 m DTT, 0.04% bromphenol blue and 35% glycerol
   7.5% SDS gels (Laemmli or Tris-tricine gels, both are available commercially)
   Electrophoresis buffer
   Coomassie brilliant blue R250 stain: 0.1% Coomassie R250; 10% acetic acid, 45% methanol.

Electro-transfer unit
Electro-transfer buffer: 10 mM MES, pH 6.0, 10% MEOH and 0.01% SDS or Towbin buffer (1979): 15.6 mM Tris, 120 mM Glycine, pH 8.3, 10% MEOH and 0.01% SDS.
2 Whatman 3mM (or equivalent) paper cut to the dimensions of the gel.
PVDF membrane (Immobilon P from Millipore or others)
TBST: 10 mM Tris-HCl, pH 7, 150 mM NaCl, 0.05% Tween
Blocking solution (blotto: 5% carnation powdered milk in TBST)
Anti-Vtg antibody to be tested (polyclonal or monoclonal)
Specific secondary antibody-linked to alkaline phosphatase (either antirabbit or antimouse, depending on the source of primary antibody being used)
Substrate: bromochloroindolyl phosphate nitro blue tetrazolium

2. Sample preparation: Dilute 10 μl of each of the samples: Plasma containing Vtg (E_2-induced male plasma or plasma from a vitellogenic female) and control male plasma by adding 250 μl 2X LSB and 240 μl water. The final dilution will be 1:50. Heat samples to 90°C for 15 min and load 10 μl into wells.

Note: One can use either Laemmli gels (Laemmli, 1970) or Tris-tricine gels (Schagger and von Jagow, 1987) for electrophoresis. These gels can be purchased ready to use from a number of different vendors, for example, BioRad or Novex.

3. Loading wells of SDS-PAGE gel
Well 2—10 μl of MW markers (Amersham, rainbow) in LSB
Well 3—10 μl of Vtg plasma
Well 4—10 μl of control male plasma
Well 6—10 μl of MW markers (Amersham, rainbow) in LSB
Well 7—10 μl of Vtg plasma
Well 8—10 μl of control male plasma
4. Place gel in electrophoresis unit, add electrophoresis buffer and run gel according to the manufacturer's instructions till the bromophenol blue band reaches the bottom.
5. Remove the gel from the unit. Cut the gel in half. Place $\frac{1}{2}$ in Coomassie blue stain to visualize the proteins.
6. Immediately assemble electrotransfer sandwich following the manufacturer's instructions for the unit. Prewet PVDF membrane in 100% MEOH, then equilibrate with electro-transfer buffer. Assemble unit so that the gel PVDF membrane sandwich is between two Whatman papers and so the gel is on the side of the ( ) electrode while the PVDF membrane is on the side of the ( ) electrode.

Note: There are a number of different membranes that are used routinely for western blot analysis, including nitrocellulose, polyvinyledenedifluoride (PVDF) and nylon membranes. We prefer to use the PVDF membranes because they have a very high protein binding capacity and have been proven to be superior for this type of analysis. There are a number of manufacturers that supply these type of membranes including Millipore (Immobilon P), BioRad, Applied Biosystems and others. We have not found particular differences among the membranes. PVDF membranes require an organic agent to help wet them before being placed in the transfer buffer. Omission of this step will guarantee a bad electrotransfer.

7. Add electro-transfer buffer to unit and start the process. Follow the recommended instructions by the manufacturer. We use 3 hr at 90 Volts or 16 hr at 30 Volts.

Note: There are many different electrotransfer buffers that work well including 15.6 mM Tris, 120 mM Glycine, pH 8.3, 10% MEOH (Towbin et al., 1979) and 10 mM MES, pH 6.0, 10% MEOH. Add 0.01% SDS to the buffer to improve the transfer of vitellogenin out of the gel and onto the membrane. It is important to optimize the electrotransfer step from the gel to the membrane. This can be monitored by staining the gel with Coomassie blue after transfer and comparing it to a control gel not electrotransfered to determine whether all of the high molecular weight proteins have been released from the gel.

8. After transfer, carefully disassemble unit, taking care to keeping the membrane wet. Rinse membrane in distilled water and then in TBST.

9. Immunoblotting—Immediately place membrane in blocking solution. Let sit a minimum of 1 hr.

Note: In order to determine which of the proteins are immunoreactive with the antibody it is important to first block all protein binding sites on the membrane with a nonspecific protein and then use the antibody to bind only to the specific antigen. It is important to dilute the primary antibody to the appropriate concentration, which depends to a certain degree on the method that will be used for visualization of the final product. For alkaline phosphatase colored reactions, antibodies are generally diluted in the range of 1 thousand to 5 thousand, as empirically determined. However, for chemi-illuminescent detection, the dilutions can be much greater since these detection systems are more sensitive. In this case, antibodies are generally diluted 1 to 10,000 or 50,000 and secondary antibodies are diluted in the same range. Monoclonal antibodies need to be more concentrated than polyclonal antibodies because they are directed against a single epitope. The required conditions need to be determined empirically.

10. Rinse membrane with 2 changes 10 min each of 200 ml TBST.
11. Add primary antibody diluted to final concentration in blocking solution. Let incubate for at least 2 hr at room temperature or overnight at 4°C.

Note: For **polyclonal antibodies**: dilute antibody 1:5000—use 20 μl serum into TBST to make a final volume of 100 ml. For **monoclonal antibodies**: dilute tissue culture supernatant 1:10 in TBST—or make a 100 ml solution of 5 μg purified mAb ml of TBST.

12. Rinse membrane 10 min each with 4 changes of 200 ml TBST.
13. Add 100 ml secondary antibody-linked to alkaline phosphatase, diluted 1:1000 in TBST.

Note: Make sure you have selected the correct second antibody. Let incubate for a minimum of 2 hr at room temp or overnight at 4°C.

14. Add substrate and watch development. When bands appear, remove substrate, rinse blot 2X with TBST, and stop with 20 mm EDTA, pH 7. Rinse with water.

3. Assays for vitellogenin determination

Vitellogenin induction is a sensitive, in vivo assay that can be used to measure exposure to estrogen or estrogen mimics in oviparous animals. In females, undergoing vitellogenesis, Vtg concentrations are normally withing the 10–20 mg ml range, depending on the species. At these concentrations, it is really easy to measure. In males, however, Vtg induction, as a consequence of exposure to environmental levels of contaminants is much lower—100 ng to 100 μg range. In some highly contaminated environments, one can sometimes measure Vtg in the low mg range in males (but this is not the rule, rather it is an exception).

As indicated above, both polyclonal and monoclonal antibodies can be used to advantage to quantify Vtg in plasma samples. Polyclonal antibodies recognize many epitopes, both around the periphery and within the Vtg molecule. For this reason, the antibody is very sensitive, responding well to low levels of Vtg in males. This property, however, is also a disadvantage. It is seldom that a plasma sample collected in the field contains intact Vtg, rather one gets a mixture of intact and degraded material, with fragments in the 10–100 kDa range. This degradation may occur during sample collection or simply during storage. As the sample degrades, some epitopes recognized by the polyclonal antibody are destroyed, others normally buried inside the Vtg molecule are exposed, changing the overall reactivity of the antibody towards Vtg. Monoclonal antibodies, on the other hand, are to single epitopes. This attribute makes their use more constant, since the antibody only measures one epitope, present either in the whole molecule or in the degraded pieces. Of course, severe degradation of the sample will eventually interfere with the binding of monoclonals as well. Because there is but one epitope, monoclonal antibodies are less sensitive than polyclonals.

3.1. Collection of plasma from field samples

It is always more difficult to collect samples out in the field than it is to collect plasma from laboratory animals. The field collection protocol is not much different, however, than the protocol described above for the collection of plasma from E_2-induced animals. Samples to be collected from

the field should follow as closely as possible the following protocol.

1. Reagents and Materials
   ~~Syringes (1–5 ml)~~
   Vacutainer needles and holder (22 Ga-1, Becton Dickinson 800-631-0174)
   Vacutainers (heparanized, 3 ml, Becton Dickinson 800-631-0174)
   MS-222 (tricaine methane sulfonate)
   Labels (tough tags USA Scientific Plastics)
   Microcentrifuge tubes (0.5 ml)
   Protease inhibitor—aprotinin (Sigma A-6279) make a stock solution 10 KIU μl in 150 mM NaCl.
2. Lightly anesthetize animals with 50–100 ppm MS-222.
3. Insert needle into a vein (caudal vein for fish), insert heparinized vacutainer, collect blood, add aprotinin to a final concentration of 10 KIU ml, gently mix blood with heparin by inverting 2 times and then keep cool at 4°C. Euthanize fish after collection.
4. Centrifuge blood for 15–20 min at 4,000 × G at 4°C to obtain plasma.
5. Transfer plasma into a separate labeled microcentrifuge tube.
6. Store at 80°C until assayed.

3.2. *Determination of vitellogenin by direct ELISA*

The idea behind this assay is to quantify Vtg by binding it directly to a solid support, the surface of a microtiter well, and then detecting how much is bound with a specific antibody to Vtg. Quantitation can be achieved either directly by radiolabeling the antibody or crosslinking it to an enzyme that can be used in enzyme-linked immuno, assays, or indirectly by using a second antibody linked to an enzyme that is specific for the first antibody. Protocols for labeling antibodies can be found in most Antibody laboratory methods books (Harlow and Lane, 1988). There are now several commercial sources for antirabbit or antimouse enzyme-linked antibodies of the IgG class. Using one of these antibodies is by far the easiest method and the one described in this protocol. Quantitation of bound antibody is proportional to the amount of Vtg bound to the surface of the microtiter wells. The amount of Vtg present in a sample is determined through the use of a standard curve.

1. Reagents and Materials
   ~~96 well polystyrene microtiter plates (Nanc)~~
   Phosphate buffered saline (PBS): 0.01 M $NaPO_4$, 0.15 M NaCl, pH 7.4
   Blocking solution: 10% BSA in TBST with 0.02% sodium azide
   Tris buffered saline Tween (TBST): 10 mM Tris-HCl, pH 7, 150 mM NaCl, 0.05% Tween.
   Alkaline Phosphatase Substrate Buffer: 30 m carbonate mm diethanolamine, pH 9.6, 2 mM $MgCl_2$.
   Substrate for Alkaline Phosphatase: Dissolve 10 mg of p-nitro-phenyl phosphate (PNPP) in 10 ml of substrate buffer.
   Stop Solution for Alkaline Phosphatase: 3N NaOH.
   Microplate washer (BioRad, model 1550).
   Secondary antibody—goat antimouse immunoglobulin conjugated to alkaline phosphatase, diluted 1:1000.
   Microplate reader (BioRad, model 3550).
2. Prepare Vtg standards for quantitation purposes. Defrost 1 tube of purified Vtg, and prepare standards by serially diluting the stock solution in TBST to give a range from 10 to 1,000 ng ml. A second set of standards is prepared using diluted male control plasma equivalent to the dilution of the samples. Make sure that control Vtg is in solution. For interassay control, choose a standard female plasma sample and dilute to the required concentration to fall within the standard curve. This sample is used in every assay at the desired concentration to ensure that the assay is operating correctly. This sample can be prepared in quantity and stored in aliquots in 50% glycerol in the 20°C freezer.

Note: It is important to use a standard curve of purified Vtg prepared to be as much like the samples as possible.

3. Prepare dilutions of plasma samples. Samples should be diluted at least 1:200 in PBS to avoid interference from concentrated plasma proteins. For males, this dilution is a starting point. For females, the first dilution should be 1:1000 in PBS.

$F(ab')_2$ (Pierce) for polyclonals diluted to the appropriate concentration in blocking solution.

2. Purify the primary antibody to remove other competing proteins. If the antibody is monoclonal, then it should be purified by chromatography on Protein G to remove tissue culture proteins that would compete for binding to the microtiter plates. If the antibody is polyclonal, it is best to use affinity purified antibodies (i.e., purified by chromatography on a Vtg-bound column).
3. Bind 100 μl of the primary antibody to the bottom of each well of a microtiter plate. The antibody should be at a concentration of 10 μg ml in PBS. Incubate for 2 hr at room temperature or overnight at 4°C.
4. Wash the plate two times with PBS.
5. Add 200 μl blocking solution to each well to make sure that all the binding sites are saturated prior to adding the Vtg sample. Incubate for 2 hr at room temperature or overnight at 4°C.
6. Wash the plate two times with PBS.
7. Prepare standard curve. Defrost 1 tube of purified Vtg to prepare standards by serially diluting the stock solution in TBST to give a range from 10 to 1,000 ng ml. Prepare a second set of standards in diluted male control plasma equivalent to the dilution of the samples. Make sure that control Vtg is in solution. For interassay control, choose a standard female plasma sample and dilute to the required concentration to fall within the standard curve. Store the interassay control in 50% glycerol at 20°C.

Note: It is important to have both a standard curve of purified Vtg that is prepared to be as much like the samples as possible and a plasma sample of known Vtg content that can be used in every assay to make sure there is low interassay variability.

8. Prepare dilutions of plasma samples. Samples should be diluted at least 1:200 in blocking buffer to avoid interference from concentrated plasma proteins. For males, this dilution is a starting point. For females, the first dilution should be 1:1000 in PBS.
9. Add protein samples to the wells of the microtiter plate. Incubate for 2 hr at room temperature or overnight at 4°C.
10. Wash the plate 4 times with PBS.
11. Add the second antibody. This antibody should be in excess. Dilute the second antibody in blocking buffer. Incubate for 2 hr at room temperature or overnight at 4°C.

Note: The second antibody can be either a second monoclonal antibody that recognizes a different epitope or a rabbit polyclonal antibody that recognizes multiple epitopes. The monoclonal antibody can be labeled with enzymes or biotin. Alternatively, the second antibody can be disclosed with yet a third, commercially available antimouse or antirabbit enzyme-linked IgG.

12. Wash the plate 4 times with PBS.
13. If a third antibody is to be used, repeat steps 11 and 12 with this antibody.
14. Prepare the substrate and add 100 μl to each microtiter well.
15. Incubate for 10–30 min at room temperature.
16. Add 100 ul of stop solution to stop the development process. Positives should appear bright yellow. Read the plate at 405 nM. A plate reader is very useful for this step.
17. Standard curves are constructed by plotting the A_{405} mn of the standards versus the amount of Vtg in the standards. Concentrations of Vtg in the unknown samples are determined directly from the curve. As with all assays, this one must be validated for the species of interest.

3.4. Quantitation of Western blots by a chemi-illuminescent procedure (see Section 2.6: Validation of antibody preparation by Western blot)

As discussed above, the western blot method is very specific and gives information on the degree of degradation of the sample. This method, however, is a lot more time consuming than ELISA and is limited in the number of samples that can be analyzed. The best way to quantify the reaction is to use a chemi-illuminescent method. Several are now on the market and they all work well. The protocol described below is for Amersham's ECL method.

1. Reagents and Materials
   Electroblot of samples on a PVDF membrane
   Blocking solution (blotto: 5% carnation powdered milk in TBST)
   Anti-Vtg antibody to be tested (polyclonal or monoclonal)
   Specific secondary antibody-linked to alkaline phosphatase (either antirabbit or antimouse, depending on the source of primary antibody being used)
   Substrate: follow kit protocol
2. Follow the procedure outlined in Protocol 6, with the following changes:
   Primary antibody should be diluted 1:10,000 in blocking buffer.
   Secondary antibody should be diluted 1:10,000 in blocking buffer.

Note: The antibody should be free of azide for this assay since azide interferes with chemi-illuminescent detection.

4. Validation of assays and statistical considerations

All assays should be validated for the species of interest. Polyclonal antibodies for Vtg don't normally cross-react widely with other species. There are some monoclonals, however, that do show wide cross reactivity (Denslow et al., 1997a; Denslow et al., 1997b; Heppell et al., 1995). The antibody must be validated with purified Vtg and used with a standard curve of Vtg of the species of interest.

Parallelism of multiple standard curves (n 4) is verified by analysis of covariance to indicate that there is no significant difference between slopes. If an antibody is to be used for multiple species with one standard curve, then it is critical to show that the antibody response is parallel from one species to the next and that the correct readings are obtained from the assay.

Intrassay variability should be measured for each experiment as a coefficient of variation for replicate measurements for at least triplicates.

Interassay variability should be evaluated in the same manner for a sample that is used in several assays (N 4). Use a sample concentration that will fall roughly midway within the standard curve.

Statistical analyses should be performed using any standard statistical software package. Statistical significance at the p 0.05 is acceptable.

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